Stream 1.3: Microbial modulation of wine composition to increase wine value

1. Abstract:

In this research stream, novel wine yeast and improved fermentation management strategies were developed, with the goal of assisting Australian winemakers to achieve their targeted wine styles. The importance of wine yeast choice and significance of nitrogen supplementation were demonstrated through a series of case studies.

New yeast strains, or blends of yeast strains, were developed that either: minimised wine faults such as hydrogen sulfide or volatile acidity production; enhanced a target flavour profile, for example through optimised release of tropical aroma compounds; or generated a distinctive flavour profile that might be considered more complex. Several of these yeasts or yeast blends were made widely available to Australian winemakers in partnership with yeast suppliers.

Significant progress was made towards the development of fermentation strategies for production of lower alcohol wine, drawing upon methodologies and materials developed in Stream 2.1. Genetically modified as well as classically derived yeasts that produce up to 3% less alcohol (v/v) were generated. Given the current trend towards lower alcohol wines, these successful strategies and experimental strains represent a valuable resource for further low alcohol yeast strain selection and optimisation of their flavour profiles.

New knowledge concerning genes important in flavour compound formation was generated. A novel link between the biosynthesis of volatile yeast metabolites and ergosterol synthesis was discovered, along with a large number of genes involved in formation of hydrogen sulfide from amino acids and glutathione. These results provide a foundation for further novel yeast strain development.

2. Executive summary:

The flavour of fermented beverages such as wine owes much to the main yeast species used in their production, *Saccharomyces cerevisiae*. Where once the role of yeast in fermented beverage flavour was thought to be limited to a small number of volatile esters and alcohols, the discovery that wine yeast release highly potent sulfur aroma compounds from non-volatile precursors found in grapes reiterated that choice of yeast can signficantly influence wine style. Similarly, prior research had demonstrated that fermentation management practices can alter the wine yeasts' tendency to produce volatile compounds, which can modify the flavour profile of wine.

Consequently, this stream sought to generate novel and improved non-genetically modified (and prototype genetically modified) wine yeasts and winemaking strategies, in order to provide Australian winemakers with enhanced options to create wines with distinctive appearance, aroma, flavour, mouth-feel, and lower alcohol content. Provision of new yeast strains and enhanced advice concerning fermentation nutrients represents a relatively low cost, but high impact, opportunity for winemakers to shape wine style and to quickly respond to market demands.

To efficiently deliver these objectives, a multi-pronged approach was taken. Firstly, a large number of wine samples made with different yeasts and/or different nutrient treatments were subjected to comprehensive chemical and sensory analyses, including in some instances consumer sensory analysis. This enabled yeast strain and nutrient-related wine sensory outcomes to be mapped to chemical compounds, framed by the consideration of whether these differences mattered – could consumers perceive the difference? Secondly, yeast strains were developed using several methodologies, such as: chemical mutagenesis, breeding, and recombinant genetic modifications (GM). The latter was used to develop model wine yeast, which in turn guided non-GM strategies to achieve the same end-points.

Prototype wine yeast which produce up to 3% less alcohol (v/v) during fermentation were generated through genetic modifications. Delivery of these prototypes drew upon 'omics tools developed in

Stream 2.1, accompanied by an Australia-wide collaboration focused on the study of wine fermentation as a holistic 'system' – in other words 'systems biology'. In this approach, many components of a system are studied simultaneously, rather than using traditional reductionist approaches where a component is analysed in isolation. Notably, this systems biology approach led to the identification of complex pathways and regulatory elements which allowed partial redirection of yeast metabolism 'away' from ethanol production.

A range of non-GM wine yeast were subsequently generated which produce between 1% and 3% less alcohol (v/v). At this stage, these experimental yeasts produce higher levels of some undesirable flavours and require further optimisation, or development, in parallel with technologies that minimise the impact of those undesirable flavours.

Genetic modification also served to deliver new knowledge of how wine yeast produce flavour compounds. Specifically, a novel link was discovered between the biosynthetic pathways of yeast sterols and production of esters, alcohols and volatile fatty acids. Drawing upon yeast deletion libraries, the AWRI Culture Collection, and 'systems biology' approaches described above, many new genes involved in hydrogen sulfide formation and regulation could be identified. Some overlap was found between hydrogen sulfide formation and release of volatile sulfur compounds associated with tropical flavours such as 'passion-fruit' from their non-volatile grape-derived precursors.

Novel industry-ready wine yeasts were developed that: produced less hydrogen sulfide; produced less volatile acidity; produced higher concentrations of some esters; and yielded wines with more diverse flavour profiles. In addition, the desirable properties of existing yeast strains were harnessed through co-inoculation to deliver enhanced sensory properties in Sauvignon Blanc.

New knowledge on how nitrogen supplementation of nitrogen deficient grape musts impacts on wine flavour profile and style was generated, despite a relatively long history of inorganic nitrogen usage (with diammonium phosphate: DAP) in wineries globally. In addition to affecting fermentation duration and hydrogen sulfide production, DAP addition significantly affected the formation of esters, alcohols, volatile fatty acids and volatile sulfur compounds; irrespective of grape variety, aroma profiles were found to vary according to yeast strain. Notably, in some cases studied, moderate supplementation unexpectedly caused specific wine yeast strains to produce higher levels of hydrogen sulfide, showing the complexity of sulfur metabolism amongst wine yeast. Excessive supplementation with DAP nitrogen but not amino acid nitrogen (organic nitrogen), as present in grapes from highly fertile sites, led to increased production of sensorial 'volatile acidity' or 'estery' taint, which was attributed to excessive ethyl acetate production. This observation provides a rationale for occurrence of ester taint in finished wine which can result from excessive use of DAP in rescue of stuck ferments. Proprietary nutrient preparations generally had more subtle impacts on production of aroma compounds, including hydrogen sulfide, due to their relatively low nitrogen content. Taken together, and considering the sensory impact and economic cost of nitrogen supplementation versus the risk of sluggish fermentations, a case for grape must nitrogen measurement and guided nitrogen supplementation was made.

The outcomes of this stream were embodied in a range of commercially available yeast strains, and the broad communication to industry through all available means. Of particular note is the production of the low-alcohol fact sheet, which summarises a range of strategies, and particularly their combination, for production of lower alcohol wines.

Additional value will be derived beyond the completion of this stream as learnings are adapted and and adopted. Some of the knowledge generated will be drawn upon in future yeast strain development activities, or could be packaged together to provide winemakers with multiple options to achieve particular style-related outcomes, such as reducing levels of volatile acidity.

Affiliation	Area of support/contribution
Flinders University	Expression of CS-lyase proteins and their characterisation
University of Western Sydney	Alternative pathways for yeast formation of H_2S

Affiliation	Area of support/contribution
Laffort Australia	Alternative pathways for yeast formation of H_2S
AB Mauri	Development and commercialisation of novel yeast
Universidad San Sebastian	Expression of genes responsible for the formation of flavour active compounds
Bioplatforms Australia Ltd	Funding for Wine Yeasts Systems Biology initiative
Macquarie University	Proteomics support through Wine Yeasts Systems Biology initiative
Australian Proteome Analysis Facility	Proteomics support for the Wine Yeasts Systems Biology initiative
University of Melbourne	Metabolomics support through Wine Yeast Systems Biology initiative
University of New South Wales	Informatics support for Wine Yeast Systems Biology initiative
Universidad Rovira & Virgili	Ethanol production by non-*Saccharomyces* yeast strains
Queensland University	Fluxomics and mathematical modelling of wine fermentations
Stellenbosch University	Development of GM low ethanol wine yeasts
Australian Genome Research Facility	Provision of genomic and transcriptomic sequencing
Cool Climate Oenology and Viticulture Institute, Brock University, St Catharines	Fermentation trials of low acetic acid-producing *S. cerevisiae* x *S. bayanus* hybrids
The Yalumba Wine Company	Hybrid yeast wine trials in different grape varietals
Oliver's Taranga Vineyards	Hybrid yeast wine trials in different grape varietals
Frogmore Creek Wines	Cool climate Pinot Noir hybrid yeast wine trials
Barwick Estates	Pinot Noir trials using hybrid wine yeast
Department of Genetics, Stanford University	Ultra-high throughput sequencing to identify differences in genomic expression of *Saccharomyces* interspecific hybrids; Genomic analysis of *Saccharomyces* interspecific hybrids; DNA chip-based genomic analysis of *Saccharomyces* interspecific hybrids
Tscharke Wines	Winemaking trials
Victoria University	Yeast ethanol-stress tolerance
China Agricultural University	Variation in wine yeast tolerance to low pH must
Orlando Wines	Characterisation of wild ferments; Provision of low YAN grapes

3. Background:

This stream sought to generate novel and improved non-genetically modified (and prototype genetically modified) wine yeasts and winemaking strategies, in order to provide Australian winemakers with enhanced options to meet the demands of an ever-changing, wide diversity of consumer preferences for appearance, aroma, flavour, mouth-feel, and alcohol content in wine.

Previous work at the AWRI and other research organisations had shown that choice of yeast and fermentation processes affect the composition of wine in a significant way, impacting not only on ethanol content but also on wine style. Thus, microorganisms and fermentation processes provide readily available approaches for modulating wine sensory attributes, even within a given variety and without the need for major changes in processing hardware. Furthermore, the well known sub-optimal nutrient content of grapes provides the opportunity to evaluate the impacts on wine style of nutritional supplements, which are normally used for enhancing production efficiency.

Development of novel non-genetically modified (non-GM) yeast strains for immediate adoption by industry, provided winemakers' with options to diversify their wine styles, or meet target specifications. At the same time, genetically modified yeasts were generated, largely to enhance knowledge; genetically modified yeasts will only be made available to the industry if and when regulatory requirements are met and consumer opinion and industry policy are accepting of genetically modified organisms.

The expected outcomes of this stream were:

- Delivery of novel wine yeast strains that produce lower levels of ethanol than existing wine yeasts.
- Delivery of novel wine yeast strains that impart diverse and improved sensory attributes to wine.
- Delivery of novel inoculation strategies and optimised nutrient management for increasing the diversity of wine composition and sensory attributes.
- Risk factors for suboptimal fermentation identified and strategies for risk management developed and communicated to industry (introduced in July 2011).

4. Stream objectives:

The objectives for this stream, as listed in the 7-year funding agreement were to provide winemakers with:

- novel yeast strains that enhance product differentiation to enable the delivery of wines that meet consumer preferences; and
- new knowledge on how to manage yeast in wine fermentations to produce wines of high consumer preference.

Changes to this stream were implemented in July 2011 to finalise, ahead of schedule, research into management of fermentation nutrients to meet wine composition and sensory specification. In its place, combined with resources previously assigned to generation of robust non-GM yeast strains (Stream 2.1), new outputs and milestones were established. This shifted the focus onto understanding the impact of interactions between yeast strains and grape juice/must composition on fermentation performance and wine style.

5. Methodology:

Delivery of both stream objectives was heavily reliant upon capacity to relate chemical composition of wines made using different yeast strains and/or nutrient treatments, to sensory profiles and ultimately consumer preferences. A large number of winemaking trials were performed, ranging from laboratory-scale and replicated small-lot winemaking (in conjunction with Provisor and WIC Winemaking Services), through to several thousand litre commercial-scale fermentations conducted by collaborating wineries. Depending upon the objectives of the specific winemaking trial, chemical composition of wines were assessed in collaboration with Streams 1.1 and 1.2, or through Metabolomics Australia's AWRI node. Formal sensory studies were conducted through Stream 3.1 utilising replicated small-lot wines. Consumer sensory studies were performed (Stream 3.1) using a small number of these sample sets, in order to assess whether yeast strain impacts were sufficient to affect consumer preferences. Critical aspects of new analytical chemistry and sensory methodologies are described in the relevant stream reports.

Correlations between sensory attributes and chemical composition of these experimental wines (cf. above) provided guidance for targeted yeast strain development activities. For example, early results concerning strain impact on Sauvignon Blanc flavour, combined with winemaker's observations, highlighted that yeast strains commonly used to make Sauvignon Blanc in Australia were not unlocking the full flavour potential available in grape musts. Furthermore, Sauvignon Blanc from New Zealand was increasing in popularity with consumers at the time, emphasising the need for Australian winemakers to boost the aromatic intensity of their wines.

Further guidance in targeted yeast strain development activities was gained through systems biology and 'omics experimental approaches, including the wine yeast gene deletion library, described in Stream 2.1. Systems biology[1] was applied to generate detailed knowledge of wine yeast central carbon metabolism. Transcriptomics[2] was used to generate candidate genes involved in flavour compound formation for further investigation, harnessing gene deletion strains provided by Stream 2.1.

Targeted yeast strain development then proceeded along parallel lines to deliver novel genetically modified strains, as well as industry-ready non-GM solutions. The method by which most GM strains were constructed involved two steps, whereby the modification was introduced into the yeast chromosome and then extraneous DNA (including antibiotic markers) were removed. This 'clean' form of genetic modification yielded, where relevant, 'self-cloned' yeast strains that contained only DNA originating from the wine yeast *S. cerevisiae* – such 'self-cloned' GM strains are likely to be the first considered acceptable for use by the Australian industry, and in some regions of the world are not classified as GM organisms.

Development of non-GM strains was guided by successful GM implementation of strategies to achieve a given target, i.e. manipulation of gene x resulted in the desired yeast characteristics and therefore all scientific literature relating to gene x was examined to identify selective pressure that would allow isolation of variants carrying a similar variation. This was particularly relevant in efforts to develop non-GM low-ethanol producing strains.

The techniques used to generate non-GM yeast strains with targeted characteristics included: random chemical mutagenesis; clonal selection from non-mutagenised yeast populations; adaptive evolution; and classical breeding. The choice of technique in each case depended upon the particular target, extent of available genetic or biochemical knowledge, and availability of yeast strains displaying natural variation for the target characteristic. Successful strain development required appropriate design of selective conditions to favour variants with the characteristics sought, or development of high-throughput screening methodologies suitable for sifting through large numbers of candidate cells generated.

This was particularly so for the development of interspecies hybrid strains for the purpose of differentiating wine style. The rationale behind this component of the stream was that introduction of new *Saccharomyces* genomes (from species not normally part of fermentation ecology), through hybridisation with *S. cerevisiae* wine strains, retained the unique fermentation properties of *S. cerevisiae* and provided completely novel strains carrying within their new genomes greater genetic diversity than exists between humans and mice. Once generated, novel hybrids required extensive molecular validation and exploration of potential winemaking applications, the latter generally achieved through collaboration with Australian winemakers.

6. Results and discussion:

Provide winemakers with novel yeast strains that enhance product differentiation to enable the delivery of wines that meet consumer preferences

Some winemakers just want a yeast that 'gets the job done' – and use the same strain for all wine styles, while others use different strains for different styles. Nonetheless, prior to this research, the impact of yeast strain on wine style was not widely recognised in industry, and the importance of yeast strain choice on wine flavour was underestimated.

To generate suitable demonstration case studies, small-lot replicated winemaking trials were conducted throughout the project . Early results showed that some yeast strains can accentuate varietal characters in Sauvignon Blanc (Swiegers et al. 2009), enhancing wine quality as perceived by winemakers. A follow-up study, where chemical composition and sensory profiles of Sauvignon Blanc wines made

1The integration of experimental datasets across multiple 'omics platforms to gain as holistic an understanding of the biological system as possible.
2 The study of all RNA 'messages' activated by an organism under the conditions being studied.

with different yeast strains were determined three years post-bottling, debunked the perception that yeast impact is short lived (King et al. 2010a, 2011a). In both young and aged Sauvignon Blanc wine, polyfunctional thiols played a critical role in determining sensory characteristics (King et al. 2010a, 2011a). Sensory differences in Chardonnay wines fermented with different yeast strains were also influenced by these tropical thiols, along with the relative production of esters, higher alcohols and volatile fatty acids (Curtin et al. 2009, Curtin et al. 2011b, a), and these differences were sufficient to impact upon consumer preferences. Interestingly, the most preferred wine from these trials was made using a blended yeast product incorporating non-*Saccharomyces* yeast species, and the overall balance of volatiles gave it a reasonable approximation of a 'wild' fermentation.

In collaboration with Stream 1.2, a preliminary study of yeast strain impact on quality indicators in Shiraz demonstrated that choice of yeast results in markedly different tannin composition and concentration – sufficient to potentially influence wine quality gradings (Holt et al. 2013).

Fundamental knowledge of yeast genes involved in flavour compound formation

The benchmarking studies emphasised that there is no single 'perfect' wine yeast strain. For example, the highest thiol-producing strain in the Sauvignon Blanc study (Swiegers et al. 2009) also led to the highest concentration in volatile acidity (VA). Hence, knowledge of yeast genes involved in formation of a broad range of flavour compounds was required to underpin novel strain development, with the goal of being able to optimise more than one trait.

The expression levels of ten candidate genes, previously identified as encoding key enzymes potentially involved in higher alcohol and ester formation, were determined during fermentation at different temperatures – a known modulator of flavour compound production (Molina et al. 2009). While temperature was shown to affect expression of these genes (Molina et al. 2007), no clear correlations could be observed between the expression of candidate genes and altered flavour profiles. Consequently, a more holistic approach was taken, where the transcriptomes of two wine yeast, which differed in their capacity to release and produce volatile aroma compounds, were compared to generate a list of ~100 genes for further study. Genes from this extended list were prioritised according to magnitude of differential expression, and 20 experimental yeast strains with single gene deletions were screened for effects on polyfunctional thiol and ester formation.Through this approach, a novel link was discovered among three genes (*ERG3, ERG5* and *ERG6*) encoding ergosterol biosynthetic pathway enzymes and formation of esters, higher alcohols, and volatile fatty acids (Kievit et al. 2010).

Novel yeast genes involved in polyfunctional thiol formation were also sought. A carbon-sulfur (CS) lyase enzyme from *S. cerevisiae* was purified and shown to have *in-vitro* activity against cysteine conjugate precursors for polyfunctional thiols 3MH and 4MMP (Holt et al. 2011, Holt et al. 2012). The gene encoding this protein, *STR3*, when expressed in a commercial wine yeast, enhanced release of 3MH during wine fermentation (Holt et al. 2011, Holt et al. 2012). At this stage, concurrent work at the AWRI (Stream 1.1) provided evidence that other thiol precursors are present in grape must, with the glutathionated forms possibly more abundant than cysteine conjugates (Capone et al. 2010). Release of 3MH from glutathionylated-3MH by wine yeast was demonstrated (Winter et al. 2011a), and the involvement of additional precursor degradation steps inferred. A novel high-throughput methodology for H_2S detection was developed (Winter and Curtin, 2012) and strains from the laboratory yeast and wine yeast gene deletion libraries (Stream 2.1) were screened. A central role of the vacuole in S-compound metabolism was identified (Winter, G. 2012).

Development of non-GM yeast strains and novel co-inoculation strategies

Efforts to develop new industry-ready yeast strains utilised breeding and mutagenesis strategies, the latter guided by knowledge of yeast genes important to the production of some flavour compounds. Mutagenesis was used to isolate variants of the widely-used commercial wine yeast strain Maurivin PDM which produced negligible amounts of hydrogen sulfide during fermentation (Cordente et al. 2009, 2011). These strains carried novel mutations in genes known to mediate the formation of

hfydrogen sulfide. A similar approach was used to obtain PDM mutants that produced less volatile acidity, whereby a novel link between the transcription factor *YAP1* and acetic acid production was discovered (Cordente et al. 2012a).

Classical yeast breeding is limited by the extent of natural genetic variation which can be drawn upon to that within a single species. The AWRI successfully developed methodologies to achieve interspecies hybridisation within the broader *Saccharomyces* clade, taking advantage of a biological process called 'rare mating', as described in Chambers et al. (2009) and Bellon (2010). This methodology was used to develop a large number of interspecies hybrids among commercial *S. cerevisiae* wine yeast strains and the species *S. paradoxus*, *S. cariocanus*, *S. kudriavzevii*, *S. bayanus* and *S. mikatae*. These novel strains impart diverse wine aroma and flavour profiles, including the production of volatiles not observed in concurrent *S. cerevisiae* fermentations (Bellon et al. 2011, 2013). Wines made with interspecies hybrids were presented and very well received during winemaker tastings in 'Winemaking with non-conventional yeast' workshops staged at the 11th and 12th Australian Wine Industry Technical Conferences held in 2007 and 2010 respectively, and a 'Taming the Pinot Noir terrior' workshop staged at the 8th International Cool Climate Symposium in 2012. In some cases, wines from hybrid yeast were judged as 'better' than equivalent wines made with industry standard yeast strains (Bellon et al. 2008). In addition to flavour diversification benefits, *S. cerevisiae* interspecific hybrids represent another option for production of wines with lower levels of volatile acidity (Bellon et al. 2013). Genome stability was evaluated during wine fermentation for one hybrid system studied. Whilst a high proportion of hybrid cells were stable throughout fermentation, some changes to genome structure occurred (Bellon et al. 2013), highlighting the need for further research in this area.

To harness full value of the research described in this stream, strategies for combining desirable traits (i.e. enhanced ester production with minimal hydrogen sulfide) were explored.

Building upon the Sauvignon Blanc benchmarking study and enhanced knowledge of genes involved in polyfunctional thiol and ester formation, co-inoculation strategies were designed to take advantage of metabolic capabilities of different yeast strains. These co-inoculations were shown to alter wine chemical composition and sensory properties in a manner consistent with consumer preferences for Sauvignon Blanc (King et al. 2008a, b, 2010b).

Successful development of novel mutants and co-inoculation strategies inspired an effort to expand sensory divergence in Chardonnay. Mutants of a commercial wine yeast, Anchor N96, were prepared by chemical mutagenesis with ethyl methanesulfonate. Strains were isolated that overproduced ethyl-hexanoate and hexanoic acid, or several acetate esters; sensory descriptive analysis confirmed the divergent sensory profiles – ranging from *cheese*-like to *tropical fruit* – imparted by these strains. A potential application of these strains is to use them in different ratios in co-inoculation, thus enabling generation of divergent sensory profiles whilst ensuring reliable fermentation.

Ideally, as many desirable properties as possible would be combined into a single strain through targeted breeding, as this would provide the simplest means of application by winemakers. To demonstrate this principle, methodology was developed to harness flavour-active properties of interspecies hybrids, and other oenological properties found in *S. cerevisiae* mutants, through further hybridisation (Bizaj et al. 2012). Whilst interspecies hybrids and *S. cerevisiae* mutants are each typically considered breeding 'dead-ends', novel hybrids were successfully generated. These advanced hybrids produced wines with similar volatile fingerprints as observed for their 'high-flavour' interspecies hybrid parent, and produced low levels of hydrogen sulfide similar to their low-hydrogen sulfide *S. cerevisiae* mutant parent (Bizaj et al. 2012). In light of results from other research in this stream, consideration of genomic stability for these proof-of-concept newly developed [hybrid x mutant] progeny is warranted.

Knowledge of yeast strain and flavour impact generated in this stream was summarised in key reviews, book chapters, and industry articles (Cordente et.al. 2012b, Curtin et al. 2011b , a, Curtin and Cordente 2013, Swiegers and Pretorius 2007, 2008).

Development of low-ethanol yeast strains and strategies to minimise alcohol levels in wine
S. cerevisiae wine yeast have evolved over millions of years to efficiently convert sugar to alcohol. Redirecting carbon to other end-points represents the 'holy grail' of wine yeast strain development – a complex task, but if achieved resultant yeast strains have the potential to revolutionise Australian winemaking.

A targeted approach drawing upon pre-existing knowledge of yeast carbon metabolism yielded a large number (60) of genetically modified wine yeast strains (Varela et al. 2012a). This work confirmed previously used modifications as effective in decreasing ethanol formation during fermentation, and ruled out as ineffective several other strategies suggested in the scientific literature. This study highlighted the need for a more systematic approach, and identified gaps in existing knowledge of wine yeast metabolism.

Drawing upon 'omics methodologies developed in Stream 2.1, a collaborative wine yeast systems biology project was initiated to address these gaps (Schmidt et al. 2012). Comparison of the wine fermentation system for previously sequenced wine yeast strain AWRI 1631 (Stream 2.1), and a version of this strain modified to produce less alcohol, revealed how wine yeast maintains a balanced redox state when carbon is directed towards glycerol rather than ethanol (Varela et al. 2010b). Further genetic modifications were introduced to minimise negative wine aroma impacts whilst decreasing ethanol concentration from 14.1% (v/v) to 11.5% (v/v).

Parallel efforts drew upon new knowledge to design non-GM approaches to the generation of low-ethanol yeast strains. The methodologies available for non-GM strain development (Chambers et al. 2009), and available microbial solutions to lowering ethanol concentrations in wine (Kutyna et al. 2010) were reviewed. Adaptive evolution was utilised to enhance glycerol production by a laboratory yeast strain (Kutyna et al. 2012). Unfortunately there was no concomitant decrease in ethanol formation, thus it was decided to discontinue adaptive evolution strategies. Instead, a mutagenesis strategy, based upon successful GM low-ethanol strain development, yielded several non-GM wine yeast strains which produce up to 3.5% (v/v) less ethanol during Chardonnay fermentation. These strains produce some negative aromas, however, and require further optimisation. In an additional non-GM approach, screening of 50 non-*Saccharomyces* yeast species from the AWRI Culture Collection (Stream 2.1) revealed a strain that, when used in sequential inoculation with an *S. cerevisiae* wine yeast, yields an approximate 1% (v/v) decrease in final wine ethanol levels (Contreras, et al., 2013).

An AWRI Fact Sheet was developed that summarised opportunities for grapegrowers and winemakers to adjust alcohol concentration of wine using a range of approaches. Emphasis was placed upon the potential for combinatorial strategies to yield higher quality low-ethanol wines, and the need to understand the impact of decisions made across the value chain. For example, a collaborative sequential-harvest study (Stream 1.2) confirmed that early harvest delivered wines that contain less alcohol, at the cost of lower production of key yeast flavour compounds (Bindon et al. 2013). Unexpectedly, the mid-harvest wines that contained up to 2% less alcohol (v/v) were preferred by consumers. Recommendations contained in the Fact Sheet were also made available to Australian winemakers through industry publications (Coulter et al. 2010, Varela et al. 2010a, Stockley et al. 2011).

Provide winemakers with new knowledge on how to manage yeast in wine fermentations to produce wines of high consumer preference

A longstanding practice in the Australian wine industry has been supplementation of nitrogen-deficient grape musts with inorganic nitrogen, in the form of diammonium phosphate (DAP). Previous research had shown DAP supplementation improves fermentation rate and can be used as a tool to modulate hydrogen sulfide formation. However, the broader impact of DAP on wine style had not been examined nor had the implications of excess DAP additions to non-deficient musts.

The impact of DAP supplementation on non-volatile wine chemical composition was demonstrated

across several fermentation studies in model juice (white: Chardonnay and Albariño and red: Shiraz) (Henschke et al. 2012a, 2012b, Torrea et al. 2011, Ugliano et al. 2007, Ugliano and Henschke 2008d, Ugliano et al. 2008b, Vilanova et al. 2007, 2012, 2013). Matrix and yeast strain dependencies were evident; for example DAP increased the formation of glycerol in some studies, but decreased it in others. It was evident that DAP caused one yeast strain to produce more glycerol and another strain to produce less, when a range of nitrogen concentrations were used in one juice (Vilanova et al. 2007). For the first time, nitrogen supplementation during fermentation was shown to impact red wine colour – leading to higher concentrations of the key anthocyanin malvidin-3-glucoside – and increased color intensity in DAP-supplemented fermentations (Ugliano et al. 2008b, Ugliano et al. 2008c).

The broader impact of DAP on wine style was comprehensively demonstrated by:

- profiling of yeast-produced volatile fermentation products (Carrau et al. 2008, Henschke et al. 2012b, Torrea et al. 2011, Ugliano et al. 2007, 2008b, Ugliano and Henschke 2008d, Vilanova et al. 2007, 2012);
- yeast-produced volatile sulfur compounds (Henschke et al. 2012b, Ugliano et al. 2007, Ugliano and Henschke 2008d, 2010c, Ugliano et al. 2009a, 2010a, b, 2012, Winter et al. 2011b);
- grape-derived varietal flavour compounds (Ugliano et al. 2008b, Vilanova et al. 2012); and
- through parallel sensory studies (Torrea et al. 2011, Ugliano 2010b).

Matrix dependencies were again evident, for example grape-derived terpenes and norisoprenoids were unaffected by DAP in red wine fermentations (Ugliano et al. 2008b), but their concentrations in white wine differed significantly in response to DAP (Vilanova et al. 2012).

DAP supplementation significantly affected the sensory profile of red and white wines (Curtin et al. 2011a, Henschke et al. 2012b, Ugliano et al. 2007, Ugliano and Henschke 2008d, Ugliano et al. 2010b; Torrea et al. 2011). Wines made by fermenting unsupplemented low nitrogen musts exhibited complex aroma profiles, whereas moderate nitrogen additions yielded wines with more intense estery, fruity and floral profiles. High nitrogen wines were dominated by 'solvent' characters caused by over-production of ethyl acetate. Correlations were made between sensory profiles and volatile flavour compound concentrations for red wines made with different yeast species and nitrogen additions. These results inferred that 'red fruit'/'dark fruit'/'confectionary' characters were associated with esters and volatile sulfur compounds (Ugliano et al. 2010b).

The long-standing notion that DAP supplementation is a tool for minimising hydrogen sulfide formation was re-visited. Regulation of hydrogen sulfide production by DAP was found to be highly strain dependent and, unexpectedly, in some cases moderate additions of DAP stimulated hydrogen sulfide formation (Ugliano et al. 2009a, b, c, 2010a, b). It has also been assumed that hydrogen sulfide measured in the head-space during fermentation is indicative of eventual levels in finished wine. Results from this work demonstrated that hydrogen sulfide production during fermentation is poorly correlated with residual hydrogen sulfide content in wine (Ugliano et al. 2009a, b, c; 2010b). Early production of hydrogen sulfide did not necessarily produce residual hydrogen sulfide in finished wine but delayed hydrogen sulfide formation was a significant risk factor.

In light of the significant and broad effects DAP supplementation had upon wine style, a comparative study was initiated (in conjunction with Stream 1.1) to investigate equivalent nitrogen management in the vineyard and winery (to achieve musts with similar nitrogen content). These approaches did not produce equivalent wines, in line with earlier work that suggested nitrogen in the form of amino acids did not have the same impact on wine composition as ammonium (DAP) (Henschke et al. 2012b, Torrea et al. 2011). These studies showed that vineyard nitrogen application or winery DAP- nitrogen addition to fermentation increased the concentration of important aroma compounds and aromatic intensity (Henschke et al. 2012b, Torrea et al. 2011).

Several results reported above highlighted the need to more systematically study yeast strain x grape

juice matrix interactions. These studies initially focused on strain performance (reported in Stream 2.1), however subsequent profiling of volatile fermentation products in wines (made by different yeast strains in model grape juice) demonstrated that pH and potassium concentrations are also likely to affect wine style, in a strain dependent manner (reported in Stream 2.1).

Oxygen, as an important yeast nutrient, was investigated for its impact during white wine fermentation on resultant yeast flavour compound profiles. Supplementation of Chardonnay fermentations with oxygen and/or lipids altered the balance of higher alcohols, esters, and volatile fatty acids (Varela et al. 2012b). More detailed work, undertaken later, investigated timing and rate of oxygen supplementation. Preliminary results suggest that impact on volatile sulfur compounds in wine depends on must clarity and that oxygen use during fermentation decreased reductive characters in red wine. Further research to corroborate these observations is being conducted in AWRI Project 3.5.3, *Formation and fate of positive and negative sulfur compounds*.

A number of effects were observed while studying the impact of oxygen on fermentation performance (Schmidt et al. 2013). The exclusion of oxygen in fermentations of clarified white juice caused stuck fermentations. In the same study, the smallest oxygen addition during fermentation resulted in the maximal response in terms of fermentation performance – additional oxygen did not lead to increasingly rapid ferments. Further work on factors that impact on fermentation performance is being conducted in AWRI Project 3.2.3, *Defining the nutritional drivers of yeast performance and matching yeast to must*.

In separate but complementary work, the effect of oxygen addition during 'wild' or uninoculated fermentation was investigated. Unlike inoculated fermentations, exposure of 'wild' ferments to oxygen did not decrease the duration of fermentation. Rather, oxygen addition to the fermenting must supported a much larger population of non-*Saccharomyces* yeast, which survived until ferment completion, along with a significantly higher residual hydrogen sulfide content. In summary, management of reductive character with oxygen in fermentation appears to be complex and requires more extensive study, which is being conducted in AWRI Project 3.5.3, *Formation and fate of positive and negative sulfur compounds*.

7. Outcome and Conclusion:

This stream aimed to develop yeast strains and fermentation management strategies for use by winemakers to modulate their wine styles. Positive and negative sensory characteristics that influence consumer liking were thus targeted, with guidance from consumer studies conducted in Stream 3.1. The stream also sought to provide winemakers with yeast strains that could be used to differentiate their wines, thereby adding value by facilitating 'premiumisation'.

A clear conclusion arising from research in this stream is that both yeast strains and fermentation management strategies involving nitrogen or oxygen have significant impacts upon wine style. The practical implications of this general conclusion are that while 'good wine is made in the vineyard', winemakers have multiple opportunities to shape their wine style before and during alcoholic fermentation.

Fundamental knowledge of yeast genes involved in flavour compound formation

Generation of new knowledge in this stream concerning 'flavour-active' yeast genes provides a stronger base for rational yeast strain development. Furthermore, identification of new functions for genes associated with non-flavour-related cellular pathways enables generation of new hypotheses concerning the biological importance of flavour compound production.

Alteration of expression levels for three genes in the ergosterol biosynthesis pathway; *ERG3, ERG5,* and *ERG6,* revealed that yeast biosynthesis of sterols affects production of esters, higher alcohols, and volatile fatty acids.

Release of the polyfunctional thiol 3MH from its cysteinylated precursor appears to be mediated by more than one CS-lyase enzyme. A self-cloned wine yeast overexpressing the native *S. cerevisiae* CS-lyase encoding gene *STR3* could be used in the future, pending acceptation of this class of GM organism, to enhance formation of polyfunctional thiols.

It was established that the yeast vacuole plays a crucial role in release of 3MH from its glutathionated precursor, in addition to release of hydrogen sulfide from cysteine. Of particular importance in vacuolar 3MH release is the gene *ECM38*. For both, desirable and negative classes of volatile sulfur compounds, genes that regulate vacuolar biogenesis are critical. Uptake of glutathionated-3MH was confirmed to require expression of the membrane transporter encoding gene *OPT1* – inferred in prior research conducted elsewhere. Practical implications of knowledge linking polyfunctional thiol and hydrogen sulfide formation are that efforts to manage 'negative' sulfur compounds during fermentation are likely to impact on formation of 'positive' tropical thiols.

Development of non-GM yeast strains and co-inoculation strategies

Methods to generate industry-ready wine yeast strains were successfully applied, in particular chemical mutagenesis and interspecies hybridisation. Adaptive evolution was trialled for the generation of non-GM low-alcohol yeast strains, but discontinued due to lack of success.

Co-inoculation was found to be an effective strategy for harnessing desirable traits of multiple yeast strains. Wine yeast blends Anchor Alchemy I and II were commercialised on the basis of co-inoculation research in Sauvignon Blanc.

Mutagenesis, when coupled with comprehensive selection and screening strategies, yielded industry-ready wine yeast strains. Yeast characteristics successfully optimised in separate strains include reduced hydrogen sulfide and volatile acidity production, and enhanced ester production. Of these, three low-hydrogen sulfide strains were patented and commercialised as Maurivin Distinction, Advantage and Platinum, and received an innovation award at Intervitis Interfructa 2010.

Five interspecific wine yeast hybrid yeasts have been licensed to Mauri Yeast Australia for production as Active Dried Yeast (ADY); AWRI 1501 (*S. cerevisiae* x *S. paradoxus*), AWRI Fusion (*S. cerevisiae* x *S. cariocanus*); AWRI 1503 (*S. cerevisiae* x *S. kudriavzevii*); AWRI 1504 (*S. cerevisiae* x *S. mikatae*) and AWRI Cerebay (*S. cerevisiae* x *S. bayanus*) (Chambers et al. 2009, Bellon 2010, Bellon et al. 2011). Two are available now as active dried yeast – AWRI Fusion and AWRI 1503, while a third (AWRI Cerebay) is currently undergoing production optimisation ahead of commercial release. The yeast strain AWRI 1503 received a 'Commendation' award at SITEVI exhibition in 2007.

Interspecies hybrids were successfully adopted by Australian winemakers to produce high quality wines, for example Oliver's Taranga Small Batch 1856 Viognier 2007 (a blend of wines made from AWRI 1503 and AWRI Fusion) – rated 95/100 by Huon Hooke, and 2010 Dawson and James Chardonnay (made using AWRI Fusion) – rated 96/100 by James Halliday.

Development of novel yeast strains and strategies to reduce alcohol content of wine

At the time this stream was initiated, it was uncertain when genetically modified wine yeast would be considered acceptable for wine production in Australia, thus GM and non-GM wine yeast that produced lower levels of ethanol were developed. The best GM and non-GM *S. cerevisiae* low- ethanol strains allow production of wine with 2-3% (v/v) less alcohol than standard wine yeast. These strains, however, produced wines with sensory defects due to production of off-flavours. An improved GM strain has been developed with some amelioration of sensory defects, providing a blueprint for further research to improve non-GM strains. In the shorter-term, a non-*Saccharomyces* yeast that can be used in sequential inoculation and yields wine with approximately 1% (v/v) less alcohol is a suitable candidate for commercialisation, contingent upon successful completion of pilot-scale trials and sensory evaluation of resultant wines.

Development of fermentation management strategies to ensure reliable fermentation

Whilst the primary objective of fermentation management is to avoid stuck and sluggish fermentations, consideration of impact on wine style provides winemakers with additional options to vary wine style and to respond to changing market demands.

Management of fermentation nitrogen strongly modulates wine composition and aroma profile and style in both red and white wines.

The demonstrated impacts of DAP on wine style strongly suggest that grape must nitrogen should be measured routinely, and evidence based additions of DAP should be made only where required rather than as 'blanket additions'.

Options for management of volatile acidity

A strength of this stream is that through parallel research efforts it has delivered multiple avenues to meet particular targets, such as minimising volatile acidity produced during fermentation. In addition to the previously mentioned successful mutagenesis strategy, several interspecies hybrids generated also produce lower levels of volatile acidity. Also, YAN measurement and targeted DAP additions will enable winemakers to minimise this fault on the basis that moderate YAN levels decrease volatile acidity production, whereas excessive DAP additions cause higher levels. Furthermore, low grape juice pH and potassium concentration have been established as stress conditions that cause yeast to produce more volatile acidity. The wine industry is now in a position to mitigate these risk factors through choice of yeast and appropriate fermentation management.

8. Recommendations:

Knowledge of novel genes with roles in the formation of various flavour compounds can best be utilised by a combination of approaches to new yeast strain development. Large-scale genome sequencing will, amongst other things, facilitate identification of novel gene variants (alleles). Bioinformatic analyses of variants and subsequent functional analysis of promising variants will generate markers for use in yeast breeding. Alternatively, chemical mutagenesis and selection strategies could be devised to target genes known to affect flavour compound formation (to be addressed in Project 3.2.2 of the AWRI's 2013-2018 R,D&E plan).

Specific applications that could draw upon knowledge generated in this stream include: development of novel yeast strains that overexpress *ERG* genes, and selection of yeast strains with altered vacuolar biogenesis with consequent modifications to volatile sulfur compound formation. An additional embodiement of knowledge concerning the link between sterol biosynthesis and flavour compound formation could take the form of advice to winemakers concerning yeast rehydration practices (to be addressed in Project 3.2.2 of the AWRI's 2013-2018 R,D&E plan).

Some practices, such as use of specific nutrients favour yeast sterol fortification which could reasonably be expected to influence wine flavour, but require further research to validate any changed practices (to be addressed in Project 3.2.3 of the AWRI's 2013-2018 R,D&E plan).

The development of information for winemakers concerning yeast stress responses will be based on knowledge of the link between *YAP1*, an oxidative stress regulatory gene, and volatile acidity production, along with the demonstration that low pH/low potassium stress leads to higher volatile acidity production.. The implications of yeast stress on wine style are currently poorly recognised and enhanced awareness would undoubtedly lead to reduced (avoidable) wine quality loss due to poor fermentation management (to be addressed in Projects 3.2.2, 3.2.3 and 4.2.1 in the AWRI's 2013-2018 R,D&E plan).

Some of novel strain-development strategies devised in this stream, used to generate 'proof-of-concept' wine yeast strains, are yet to deliver commercialisable products. For example, novel ester modulating strains, potentially unuseable in their own right, are currently being trialled in co-

inoculations to evaluate their application in a red variety. In some cases, optimisation of a single trait is insufficient to generate sufficient interest from yeast suppliers to proceed to commercialisation, an example being low-volatile acidity production. Targeted breeding to combine low-volatile acidity with other valued trait(s) (such as low-hydrogen sulfide production) might be a suitable path to capture the value of this work (to be addressed in Project 3.2.2 of the AWRI's 2013-2018 R,D&E plan). In other cases, generated strains are incompatible with supplier commercialisation pipelines. Interspecies hybrids have a reputation for being more challenging to optimise for production than standard wine yeasts. A pragmatic solution would be to ensure that any novel interspecies hybrids are made in parallel using multiple *S. cerevisiae* parental strains, thereby minimising the risk in upscaling their production, enhancing the likelihood of novel strains being made available sooner under favourable conditions for Australian winemakers (to be addressed in Projects 3.2.2 and 4.2.1 of the AWRI's 2013-2018 R,D&E plan).

Novel GM and non-GM low-ethanol yeast strains represent a highly valuable portfolio. Further research to optimise their flavour profiles will be essential for their eventual application by Australian winemakers. Similarly, the promising non-*Saccharomyces* yeast strain that can be used in sequential inoculation to yield lower alcohol wine requires comprehensive sensory validation. Making such a yeast strain available to Australian winemakers would require a commercialisation partner with experience in the production of non-*Saccharomyces* yeasts (to be addressed in Projects 3.3.1 and 4.2.1 in the AWRI's 2013-2018 R,D&E plan).

The broader impacts of fermentation management, including nutritional supplementation, upon wine style have been continuously communicated to winemakers via roadshows and industry publications. Nonetheless, on-going extension activities and the incorporation of new content will provide winemakers with a more comprehensive insight into fermentation management options, and further reinforce the importance of proactive fermentation management to ensure desirable wine style outcomes (to be addressed in Projects 4.1.1, 4.1.3 and 4.1.4 in the AWRI's 2013-2018 R,D&E plan).

9. Budget reconciliation:

Financial Year	Receipts / Income ❶	Outgoings / Expenditure ❷
Year 1: 2006/2007	$1,151,980	$1,151,980
Year 2: 2007/2008	$1,143,433	$1,143,433
Year 3: 2008/2009	$1,269,648	$1,269,648
Year 4: 2009/2010	$1,191,633	$1,191,633
Year 5: 2010/2011	$1,204,883	$1,204,883
Year 6: 2011/2012	$1,447,017	$1,447,017
Year 7: 2012/2013	$1,528,767	$1,528,767
TOTAL	$8,937,361	$8,937,361

❶ Note that the GWRDC – AWRI Investment Agreement budget was established and approved at an aggregate level, with variances to budget (i.e. annual overspends and underspends) reported and considered at that same aggregate (i.e. whole of agreement) level. The receipts / income relating to a Stream for any year therefore equate to the outgoings / expenditure within that Stream for that year, as any variances between total Investment Agreement funding received and total funds expended were considered at the whole of Agreement rather than individual Stream level.

❷ Includes a pro-rated share of Theme 5 *Executive management and administration*.

I hereby certify that this statement is true and accurate.

Signature of duly authorised representative.....

Chris Day — Group Manager – Corporate Services — 29/11/2013

Name: *Title:* *Date:*

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